

EXHIBIT 1

Regulation of glucose-dependent insulinotropic polypeptide release by protein in the rat

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Wolfe, M. Michael, Ka-Bing Zhao, Kenneth D. Glazier, Linda A. Jarboe, and Chi-Chuan Tseng. Regulation of glucose-dependent insulinotropic polypeptide release by protein in the rat. *Am J Physiol Gastrointest Liver Physiol* 279: G561–G566, 2000.—Glucose-dependent insulinotropic polypeptide (GIP) release has been demonstrated predominantly after ingestion of carbohydrate and fat. These studies were conducted to determine the effects of protein on GIP expression in the rat. Whereas no significant changes in duodenal mucosal GIP mRNA levels were detected in response to peptone, the duodenal GIP concentration increased from 8.4 ± 1.5 to 19.8 ± 3.2 ng GIP/mg protein at 120 min ($P < 0.01$). Plasma GIP levels also increased from 95 ± 5.2 pg/ml to a peak of 289 ± 56.1 pg/ml at 120 min ($P < 0.01$). To determine whether the effects of protein on GIP were due to stimulation of acid secretion, rats were pretreated with 10 mg/kg omeprazole, after which mucosal and plasma GIP concentrations were partially attenuated. To further examine the effects of luminal acid, rats were administered intraduodenal 0.1 M HCl for 120 min, which significantly enhanced GIP expression. These studies indicate that nutrient protein provides a potent stimulus for GIP expression in the rat, an effect that occurs at the posttranslational level and may be mediated in part through the acid-stimulatory properties of protein. The effects of acid on GIP are consistent with a role for GIP as an enterogastrone in the rat.

gastric inhibitory polypeptide; nutrients; acid secretion; enterogastrone

GLUCOSE-DEPENDENT INSULINOTROPIC polypeptide (GIP), a 42-amino-acid regulatory peptide, was first isolated from porcine small intestine and originally named “gastric inhibitory polypeptide” on the basis of its ability to inhibit acid secretion (3). Although the role of GIP as a physiological inhibitor of acid secretion has been questioned (16, 34), GIP has attained the status of an important metabolic hormone by virtue of its ability to enhance insulin release by pancreatic β -islet cells (8, 19, 20). It has thus been suggested that the principal physiological function of GIP may be its role in maintaining glucose homeostasis (5).

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The release of GIP into circulation has been shown primarily after the ingestion of two major nutrient stimuli, namely carbohydrate and fat (5, 20). Although the release of GIP has been well characterized, few studies have examined the biosynthesis of this peptide. Our laboratory (27) previously cloned a rat GIP cDNA from small intestine and demonstrated that a lipid-containing meal stimulates duodenal GIP gene expression. We also have demonstrated that glucose increases duodenal GIP gene expression, not only by enhancing GIP release into the circulation but at the transcriptional level as well (28). Studies examining GIP release after various protein meals have yielded conflicting results. Although some investigators (7, 22) have been unable to detect a stimulatory effect of protein on GIP release in humans, increases in circulating GIP levels have been demonstrated in response to the administration of individual amino acids (17, 25, 26). Past studies in this laboratory (31), employing a sensitive and specific RIA for measuring GIP, showed a 10-fold increase in serum GIP concentrations 15 min after the intragastric infusion of 10% peptone in dogs. The effect of protein on GIP release in rodents has not been examined previously.

In the present study, we have used the rat as a model to examine the physiological regulation of GIP gene expression in the small intestine by peptone, a protein hydrolysate. We have demonstrated that in addition to carbohydrates and lipids, nutrient protein provides a potent stimulus for GIP expression in the rat, an effect that occurs at the posttranslational level and may be mediated in part through the acid-stimulatory properties of protein.

MATERIALS AND METHODS

Animals and measurement of GIP by RIA. Male Sprague-Dawley rats (250–350 g), purchased from Charles River (Kingston, MA), were fasted overnight with access to water. After an overnight fast, rats were anesthetized with pentobarbital sodium (60 mg/kg) and submitted to midline laparotomy. A small gastrotomy was made, and Tygon tubing was

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passed through the mouth and into the stomach; this tubing was ligated distal to the esophagogastric junction. The stomach was then perfused for 0, 60, 120, or 240 min with solutions containing either 0.9% NaCl or peptone (protein hydrolysate, pH 7.0) at a rate of 10 ml/h. In separate experiments, one group of anesthetized rats was pretreated with 10 mg/kg omeprazole, a dose previously shown to abolish acid output in the rat (11), before intragastric peptone perfusion for 120 min, and another group was administered 0.1 M HCl by intraduodenal infusion for 120 min. The rats were killed at various time points. The duodenum was removed and cut longitudinally into two pieces; one half was used for RNA extraction, as described below, and the other half was boiled in 2.0 M acetic acid for 60 min. After boiling, the supernatant was removed and stored until assayed for GIP. The residual mucosa was then solubilized in 1 M NaOH, and tissue protein was measured using a modification of the method of Lowry et al. (15). Blood was obtained by intracardiac puncture and centrifuged at 10,000 g for 10 min. Serum was separated and stored at -20°C until assayed for GIP. GIP RIA of plasma samples and duodenal mucosal extracts was performed using the double-antibody method, as described previously (31).

RNA extraction. Total RNA from small intestine was extracted using the acid-phenol method of Chomczynski and Sacchi (6). Briefly, the tissue was homogenized in 4 M guanidinium isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, pH 7.0, and 100 mM 2-mercaptoethanol. The homogenates were mixed with phenol, 2 M sodium acetate (pH 4.1), and chloroform (800 µl/4.0 ml phenol) on a vortex for 30 s and iced for 15 min. The homogenates were then centrifuged at 4°C for 15 min at 4,350 g, and RNA in the aqueous phase was mixed with an equal volume of chloroform. The aqueous phase was again extracted and precipitated with an equal volume of isopropanol, after which samples were washed with 70% ethanol and dried. RNA yields were examined by spectrophotometric absorption at 260 and 280 nm, and total RNA was measured by determining absorption at 260 nm (A_{260} of 1 = 40 µg of RNA/ml).

Northern blot hybridization analysis. Northern blot hybridization analysis was done using stringent conditions [at 42°C with 50% (vol/vol) formamide and 5× sodium saline citrate (SSC) (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)]. Twenty micrograms of total RNA from small intestine were denatured in gel-running buffer (0.04 M MOPS, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.5, 50% formamide, and 6% formaldehyde) (12). The RNA was then electrophoresed on a 1.5% agarose-6% formaldehyde gel. The integrity of the extracted RNA was determined by the visualization of 28S and 18S ribosomal RNA bands with ethidium bromide staining. After electrophoresis at 10 V/cm, the RNA was transferred from the gel to a Duralon ultraviolet filter by capillary action, as described by the manufacturer (Stratagene). Hybridization was then performed using a 394-bp *BspM I-Sal I* fragment of the rat GIP cDNA that was radiolabeled with [α -³²P]dCTP, using the Klenow fragment of DNA polymerase I and random oligonucleotides as primers (Promega). The blots were prehybridized for 2 h at 42°C in 5× SSC, 10× Denhardt's solution, 50% (vol/vol) formamide, 50 mM NaPO₄, 1% SDS (GIBCO-BRL), and 10 µg/ml herring sperm DNA (Sigma Chemical, St. Louis, MO). The filters were then hybridized at 42°C for 16–24 h in 5× SSC, 1× Denhardt's solution, 50% formamide, 20 mM NaPO₄, 0.5% SDS, 20 µg/ml herring sperm DNA, and ~10⁷ cpm of probe/100-cm² filter. After hybridization, blots were washed once at room temperature in 1× SSC and 1% SDS for 15 min, once at room temperature in 0.5× SSC and 0.5% SDS for 15 min,

twice at room temperature in 0.1× SSC and 0.1% SDS for 15 min, and once at 50°C in 0.1× SSC and 0.1% SDS for 30 min. Autoradiograms were developed after exposure to X-ray film for 12–96 h at -70°C, using a Cronex intensifying screen (DuPont). GIP mRNA and GAPDH mRNA signals were quantified by laser densitometry and integration of the autoradiographic images. The latter was used to correct for gel loading (21, 28).

Statistical analysis. Results are expressed as means \pm SE of 4–6 separate experiments. Data was analyzed using two-way ANOVA (BMDP Statistical Software, Los Angeles, CA) for concentration and time and Student's *t*-test. Significance was assigned if $P \leq 0.05$.

RESULTS

Effects of peptone on GIP expression. No significant changes in plasma or duodenal mucosal GIP levels or duodenal GIP mRNA concentrations were detected during the intragastric infusion of 0.9% NaCl (data not shown). Initial dose-ranging experiments examining the effects of 6%, 8%, and 10% peptone solutions demonstrated that intragastric infusion of the 10% solution produced the greatest and most consistent increase in plasma GIP levels compared with a control (0.9% NaCl) infusion (Fig. 1). Thus a 10% solution was the concentration of peptone used throughout subsequent studies. The basal plasma GIP level was 95 \pm 5.2 pg/ml and increased to a maximum concentration of 289 \pm 56.1 pg/ml at 120 min (Fig. 2, $P < 0.01$). GIP levels in duodenal mucosal extracts also increased in response to intragastric peptone infusion from a basal concentration of 8.4 \pm 1.5 ng GIP/mg protein to a peak of 19.8 \pm 3.2 ng GIP/mg protein at 120 min (Fig. 3, $P < 0.02$). In contrast, the intragastric perfusion of peptone did not significantly alter steady-state duodenal GIP mRNA levels compared with rats perfused with 0.9% NaCl. GIP mRNA levels (expressed as a ratio of GIP mRNA to GAPDH mRNA) in fasting rats were 0.55 \pm

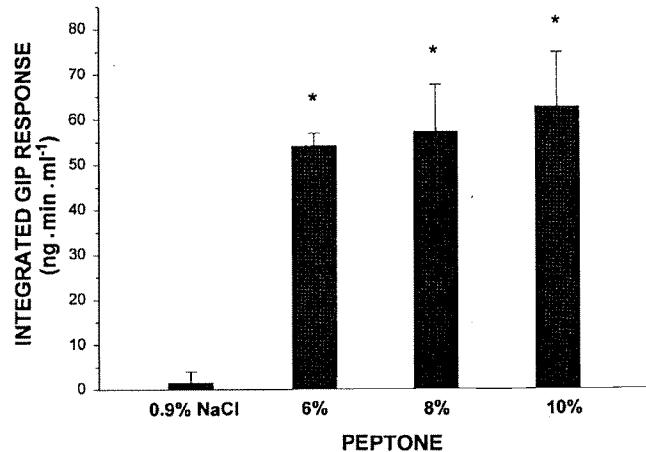


Fig. 1. Integrated plasma glucose-dependent insulinotropic polypeptide (GIP) response to graded doses of peptone administered by intragastric infusion. Meals were infused over a 4-h period, and plasma GIP levels were measured at baseline and at 30, 60, 120, and 240 min. Data are expressed as means \pm SE in ng·min·ml⁻¹ of 4–6 experiments. * $P < 0.01$ compared with control (0.9% NaCl).

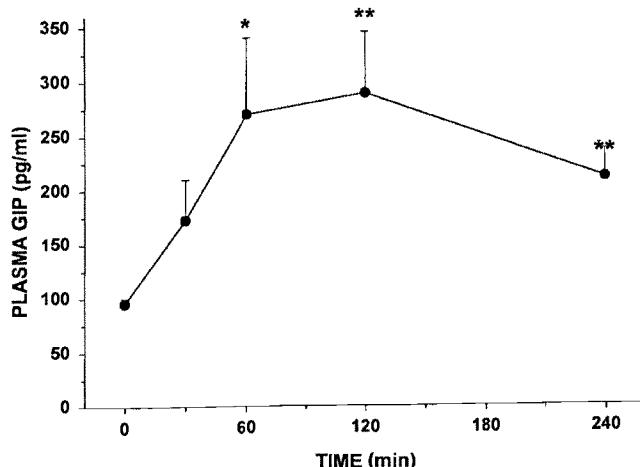


Fig. 2. Plasma GIP concentration in rats after the intragastric infusion of 10% peptone. Data are expressed as means \pm SE in pg/ml of 4–6 measurements at each time point. * P < 0.01. ** P < 0.05.

0.04 and remained unchanged in peptone-fed rats throughout the entire 4-h experimental period (Fig. 4).

Role of gastric acid in regulation of GIP expression. To determine whether the effects of protein on GIP were due to stimulation of gastric acid secretion, rats were pretreated with 10 mg/kg omeprazole for 120 min. After the administration of omeprazole, peptone-stimulated duodenal mucosal and plasma GIP concentrations were diminished. At 120 min, the duodenal mucosal GIP level decreased by ~55% in omeprazole-treated rats from 19.7 ± 3.2 to 8.9 ± 2.4 ng GIP/mg tissue protein (P < 0.02), a value not different from the basal concentration (Fig. 5A). The plasma GIP concentration after intragastric infusion of 10% peptone was 688 ± 142 pg/ml at 120 min, which was partially attenuated (~30% decrease) by pretreatment with omeprazole to 485 ± 120 pg/ml (Fig. 5B). To further examine the effects of luminal acid, anesthetized rats

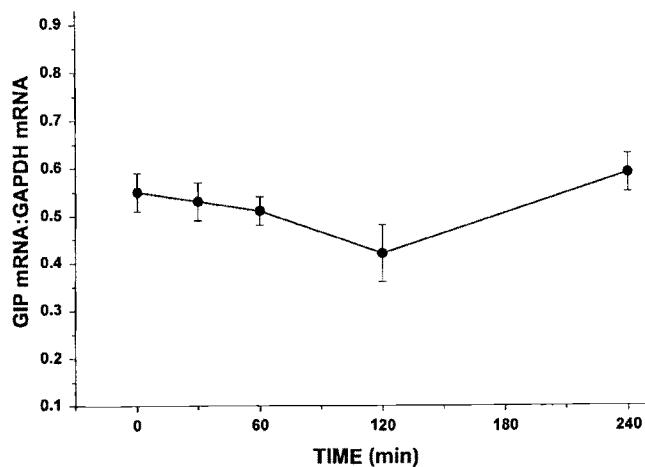


Fig. 4. Steady-state levels of duodenal GIP mRNA in rats after the intragastric infusion of 10% peptone. GIP mRNA concentration is expressed as the mean \pm SE ratio of GIP mRNA to GAPDH mRNA (to correct for gel loading). Each time point represents measurements from 4–6 rats for each group.

were administered 0.1 M HCl by intraduodenal infusion for 120 min. In response to duodenal acidification, although only a modest, but significant, increase in circulating GIP levels was seen after the intraduodenal

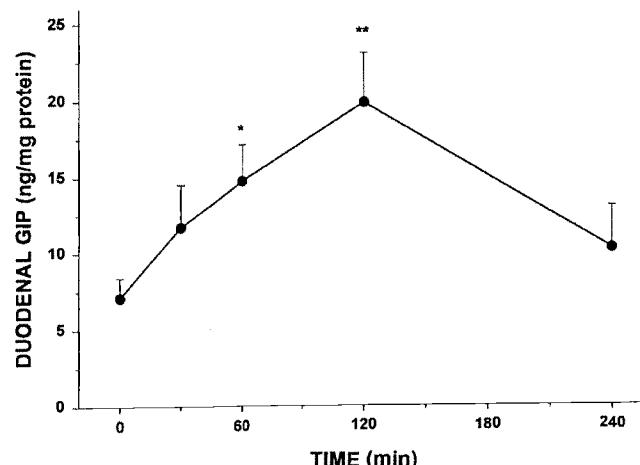


Fig. 3. Duodenal mucosal GIP concentration in rats after the intragastric infusion of 10% peptone. Data are expressed as means \pm SE in ng GIP/mg protein of 4–6 measurements at each time point. * P < 0.01. ** P < 0.02.

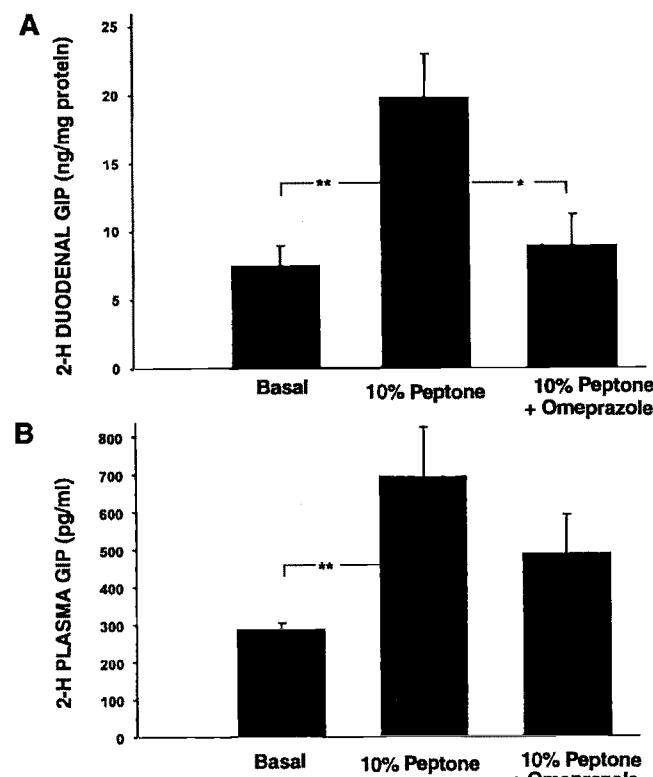


Fig. 5. Two-hour duodenal mucosal (A) and plasma (B) GIP concentrations in rats given 10% peptone by intragastric infusion in the presence or absence of pretreatment with intraperitoneal omeprazole (10 mg/kg). Data are expressed as means \pm SE in pg/ml (A) or ng GIP/mg tissue protein (B) from 10 rats at each time point. * P < 0.02. ** P < 0.01.

infusion of 0.1 M HCl, a marked and progressive increase in mucosal concentrations of GIP was detected (Fig. 6). Likewise, after 120 min of 0.1 M HCl duodenal perfusion, the ratio of duodenal mucosal GIP mRNA to GAPDH mRNA increased by ~124%, from 1.44 ± 0.12 to 3.22 ± 0.36 ($P < 0.001$) (Fig. 7).

DISCUSSION

The release of GIP into the circulation has been demonstrated primarily after the ingestion of two major nutrient stimuli, namely carbohydrate and fat (5, 20). However, the release of GIP in response to oral glucose differs in both magnitude and timing from that which follows fat ingestion. After glucose ingestion, GIP release is rapid, preceding insulin release, and reaches a peak in peripheral venous blood in ~15 min, returning to basal values by 180 min (5). In early studies, Andersen et al. (1) examined the effect of GIP on insulin release after oral glucose feeding in normal subjects using the glucose clamp technique. Andersen et al. (1) found that the stimulation of insulin secretion by GIP released after glucose required hyperglycemia. Therefore, the insulinotropic action of GIP requires a threshold concentration of blood glucose, below which

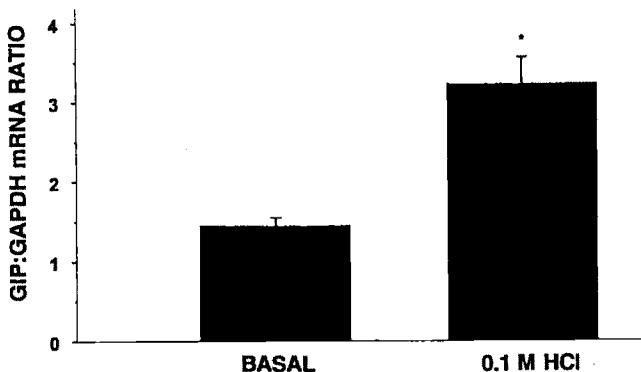


Fig. 7. Two-hour steady-state levels of duodenal GIP mRNA in rats administered 0.1 M HCl by intraduodenal infusion. Values are expressed as means \pm SE of the ratio of GIP mRNA to GAPDH mRNA to correct for gel loading. Each time point represents measurements from 6 rats. * $P < 0.001$ compared with basal values.

GIP is not insulinotropic (1, 4). After the ingestion of 100 ml of a triglyceride suspension, the peripheral blood GIP response does not reach a peak until 120–150 min and does not return to baseline by 180 min (2). Furthermore, the amount of GIP released in response to fat is greater than that which follows glucose ingestion (2).

GIP release into the circulation has previously been demonstrated in humans (7, 25, 26) and dogs (17) after the administration of leucine and other amino acid solutions. However, GIP release has not been reported in humans in response to various protein meals, including steak (7) and steamed cod (22). In addition to glucose- and fat-stimulated GIP release, we (31) previously demonstrated the release of GIP after a protein meal in dogs. These studies showed a 10-fold increase in serum GIP concentration 15 min after the intragastric infusion of 10% peptone in dogs. A small, but significant, increase in serum glucose concentrations was detected 90 min after the peptone meal, which occurred long after circulating GIP levels had returned to baseline. No changes in serum insulin concentration were detected throughout the duration of the 90-min blood collection period. The mechanisms by which amino acids and protein stimulate GIP release have not previously been examined.

The gastric phase of acid secretion accounts for ~50% of the total acid secretory response to a meal and occurs as a result of both chemical and physical factors (33). In addition to antral distension, which involves both short intramural nerve fibers and vago-vagal pathways, gastric acid secretion is stimulated by increases in intraluminal pH and by certain substances contained in food, principally protein, primarily via gastrin-mediated events (33). It has been shown that although intact proteins are poor stimulants of gastrin release and gastric acid secretion, peptic hydrolysates of the same proteins and individual amino acids are potent secretagogues (9, 13, 14). Digested protein in the duodenum also enhances acid secretion, and this duodenal phase constitutes ~5% of the total acid se-

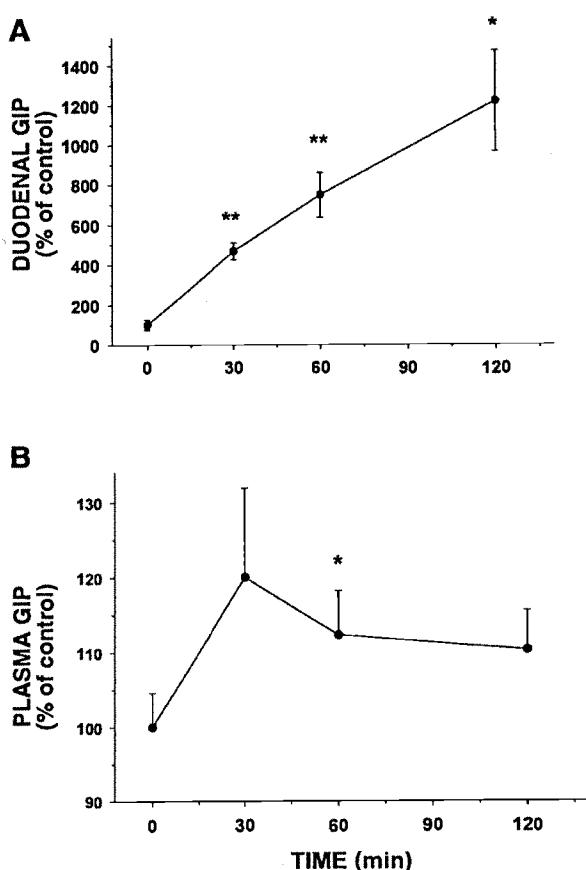


Fig. 6. Duodenal mucosal (A) and plasma (B) GIP concentrations in rats administered 0.1 M HCl by intraduodenal infusion. Data are expressed as means \pm SE (% of control) from 6 rats at each time point. * $P < 0.05$ and ** $P < 0.01$ compared with basal values.

cretory response to a meal. To explore the possibility that the peptone-stimulated GIP release in the present study was due to the stimulation of gastric acid secretion, acid output was abolished by pretreatment with omeprazole. After treatment with omeprazole, peptone-stimulated mucosal and plasma GIP concentrations were significantly attenuated (Fig. 5). Furthermore, duodenal acidification produced significant stimulatory effects on mucosal and circulating GIP concentrations (Fig. 6), consistent with the hypothesis that GIP release was mediated principally through the acid-stimulatory properties of hydrolyzed protein.

After a meal, the secretion of acid is modulated by a negative feedback mechanism in which both antral and duodenal acidification inhibits the further release of gastrin and, subsequently, acid secretion. Previous studies (18, 23, 24, 30) have shown that somatostatin derived from antral D cells inhibits the release of gastrin via paracrine mechanisms and inhibits acid secretion both indirectly by its effects on gastrin expression and by local inhibitory mechanisms in the gastric corpus and fundus. In addition to these local factors in the stomach, various regulatory peptides emanating from the small intestine, including GIP, have been proposed as enterogastrones, factors that provide additional physiological feedback inhibition of gastric acid secretion. Using antibodies to bind endogenous GIP, we (29) previously demonstrated the capacity of GIP to decrease meal-stimulated gastric acid secretion in dogs, an effect that appeared to be due to its inhibitory effects on gastrin release. Subsequent experiments in our laboratory (12), using rat antral mucosa in short-term culture, indicated that the inhibitory effects of GIP on gastrin release appeared to be mediated through the stimulation of neighboring somatostatin-containing D cells in the antrum.

In the present studies, duodenal acidification produced significant stimulatory effects on mucosal and circulating GIP concentrations (Fig. 6). However, in contrast to previous studies in our laboratory (27, 28), in which both glucose and lipid meals increased duodenal mucosal GIP mRNA concentrations, steady-state GIP mRNA levels remained unchanged in peptone-fed rats. These observations are consistent with a role for GIP as a physiological enterogastrone in the rat. Under these circumstances, the response to intraduodenal acid would be expected to produce an immediate response, with GIP release into the circulation and possibly an increase in mucosal levels occurring rapidly. Moreover, the release of GIP into the circulation did not reach peak levels until 120 min, which is significantly later than the increase observed after a glucose meal (28). Although not examined in the present study, this relative delay in GIP release may be attributed in part to the buffering effects of the peptone meal, which normally has a pH of 7.0 (31). After infusion of the peptone meal, although acid secretion would increase rapidly, the meal itself would maintain the intragastric pH at an elevated level, until endogenous gastric acid overwhelms the buffering capacity of peptone. Thus, under such circumstances, a decrease in intraduodenal

pH, and consequently GIP release, would not occur immediately, but rather would be delayed. Acid also stimulates the immediate release of mucosal and pancreatic bicarbonate, and neutralization of the acidic contents ensues promptly and persists for extended periods of time (10). As a result, the stimulus for continued GIP biosynthesis would not be present and mRNA levels could be expected to remain constant. In support of this hypothesis, the continuous duodenal infusion of 0.1 M HCl does produce a marked increase in mucosal GIP mRNA levels (Fig. 7).

In conclusion, the present studies indicate that in addition to lipids and carbohydrates, nutrient protein provides a potent stimulus for GIP expression in the rat, an effect that occurs at the posttranslational level and may be mediated in part through the acid-stimulatory properties of protein. Although the precise physiological relationship between GIP and gastric function has not been fully elucidated, it is possible that GIP may act synergistically with other candidate enterogastrones, such as secretin, enteroglucagon, and peptide YY, to inhibit antral gastrin release and acid secretion under physiological conditions (33). Further study will be necessary to fully characterize these complex interactions.

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